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Volatile anesthetics depress the depolarization-induced cytoplasmic calcium rise in PC 12 cells

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In the rat pheochromocytoma cell line PC 12, the effects of four volatile anesthetics (halothane, isoflurane, enflurane, methoxyflurane) on the K^+-evoked intracellular calcium ($[Ca^{2+}]_i$) rise were investigated using the Ca^{2+}-sensitive fluorescence dye fura-2. The $[Ca^{2+}]_i$ rise was depressed, at clinical concentrations, by all anesthetics with almost identical aqueous IC_{50} values. The study extends to neuronal cells the observation made previously in cardiac tissue that volatile anesthetics may interfere with Ca^{2+} fluxes through voltage-gated channels.

Volatile anesthetic; cytoplasmic free Ca^{2+}; Fura-2; Ca^{2+} channel; (PC 12 cell, Rat)

1. INTRODUCTION

Calcium is a key intracellular second messenger for signal transduction in nerve, muscle, endocrine and hemopoietic cells. Many Ca^{2+}-dependent cellular processes are influenced by halogenated inhalation anesthetics (e.g. myocardial contractility [1], neurotransmitter release and synaptic transmission [2], neutrophil functions [3]). Indirect evidence that volatile anesthetics affect myoplasmic Ca^{2+} control could be demonstrated at the tissue and subcellular level by electrophysiologic and radioisotope techniques [1,4]. However, direct evidence that anesthetics significantly interfere with the regulation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) level is still limited [1,5,6]. In the cat papillary muscle halothane produced a dose-dependent decrease in twitch tension and a decrease in $[Ca^{2+}]_i$ rise measured by the Ca^{2+} sensitive photoprotein aequorin [6]. Inhibition of intracellular Ca^{2+} mobilization has been observed in human polymorphonuclear leukocytes exposed to the volatile anesthetics halothane, enflurane and isoflurane [3]. In a recent report it was shown that halothane inhibited the cholinergic-receptor-mediated $^{45}Ca^{2+}$ uptake into primary cultures of bovine adrenal medulla cells [7]. Interestingly, the authors did not find a depression of the K^+-induced ^{45}Ca uptake. This was so far the only study investigating this issue in cells of neuronal origin.

The clonal rat pheochromocytoma cell line PC 12 exhibits properties of both adrenal chromaffin cells as well as sympathetic neurons [8]. In PC 12 cells the depolarization-induced transmitter release is mediated by a cytoplasmic $[Ca^{2+}]_i$ rise that is extensively characterized by pharmacological, electrophysiological and radioisotope techniques as a Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (VSCC) [9–13]. Since we recently observed that volatile anesthetics affect the K^+-evoked transmitter release from PC 12 cells (unpublished), we used this cell line to investigate the effects of the four volatile anesthetics halothane, isoflurane, enflurane and methoxyflurane on the depolarization-evoked $[Ca^{2+}]_i$

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changes by means of the Ca^{2+} sensitive fluorescence dye fura-2 [14,15].

2. MATERIALS AND METHODS

2.1. *Materials*

Halothane was obtained from Hoechst (Frankfurt, FRG), the other anesthetics were from Abbott (Wiesbaden, FRG). RPMI 1640 medium and sera were from Gibco Europe, all other buffers, media and medium supplements from Biochrom KG (Berlin). Diltiazem and verapamil were purchased from Sigma (St. Louis, MO, USA), fura-2 free acid and fura-2 acetoxymethyl ester (fura-2/AM) from Calbiochem (La Jolla, CA, USA), EGTA was from Fluka; other chemicals were of analytical grade from Merck (Darmstadt, FRG) or Sigma.

2.2. *Cell culture and fura-2 loading*

PC 12 cells (initially from Dr H. Bönisch, Würzburg) were maintained in spinner culture flasks essentially as described by [16]. The culture medium consisted of RPMI 1640 supplemented with 10% horse serum, 5% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and 2 mM glutamine. An aliquot of the cell suspension was washed by centrifugation, cell viability was assessed by trypan blue exclusion (>95% in all experiments), and cells were suspended in RPMI 1640 buffered with 20 mM Hepes, pH 7.3 (1×10^7 cells/ml), mixed with a 0.5% volume of 1 mM fura-2/AM in dimethyl sulfoxide (DMSO). After incubation at 15°C for 1 h, the PC 12 cells were washed twice, and suspended in a modified Hanks' salt solution (2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5 mM KCl, 25 mM Hepes, pH 7.3, without phenol red indicator) which served as the incubation medium for all fluorescence assays unless otherwise indicated.

2.3. *Measurement of $[Ca^{2+}]_i$ with fura-2 in the presence and absence of anesthetics*

For measurements in the presence of anesthetics the Hanks' incubation media were equilibrated by bubbling the media for 20 min at 32°C with an air stream containing the vaporized anesthetic (Dräger vaporizers, Lübeck, FRG; 0.5–5%, v/v, for halothane, enflurane, isoflurane; 0.1–2%, v/v, for methoxyflurane). The concentration of the anesthetics in the medium was checked with a gas chromatograph as described [17]. Control media were bubbled solely with air.

Before the start of each experiment the PC 12 cells were preincubated for 15 min at 32°C in Hanks' medium with or without anesthetic. The cells (4×10^6) were quickly pelleted, resuspended in 1 ml fresh medium of identical anesthetic content, and pipetted into a quartz cuvette which was tightly closed by a conical teflon plug. Assays were carried out at 32°C with a modified 4-8202-Amin-co-Bowman spectrofluorimeter (Silver Spring, MD, USA) fitted with a magnetic stirrer and a thermostatted cuvette holder. Intracellular fura-2 fluorescence intensity was measured at two quickly alternating excitation wavelengths (340/385 nm) and continuously recorded at 500 nm. KCl stock solution (2.55 M, 20 μl) and drugs dissolved in water were added directly to the cuvette. At the end of each individual trace, cells were lysed with 0.1% Triton X-100 to yield the fluorescence signals at high Ca^{2+}, followed by addition of 8 mM EGTA/50 mM Tris for minimal Ca^{2+} (<1 nM) fluorescence signals. After correction for changes of autofluorescence, $[Ca^{2+}]_i$ values were calculated according to the equation given in [14] using the 340/385 nm ratio of fluorescence intensities and a Ca^{2+}-dye dissociation constant (K_d) of 365 nM.

3. RESULTS

In the complete incubation medium containing 5 mM K^+ and 2 mM Ca^{2+} the $[Ca^{2+}]_i$ was 96 nM ± 17 nM. Raising the extracellular concentration of K^+ from 5 mM to 55 mM induced a marked rise in fura-2 fluorescence at 340 nm excitation wavelength (within 10 to 15 s), reflecting an increase in $[Ca^{2+}]_i$ from the basal level to approx. 300 nM (fig.1A,E). Within the first minute this peak concentration declined to a plateau level, which remained elevated for several minutes (fig.1). The extent of the measured $[Ca^{2+}]_i$ rise depended on the K^+ concentration, reaching a maximum (about 3-fold the resting level) above 50 mM. Similar $[Ca^{2+}]_i$ levels as shown here with fura-2 have been obtained with the Ca^{2+} indicator quin 2 in PC 12 cells [18,19].

As demonstrated in fig.1B, the K^+-induced $[Ca^{2+}]_i$ elevation was totally dependent on the

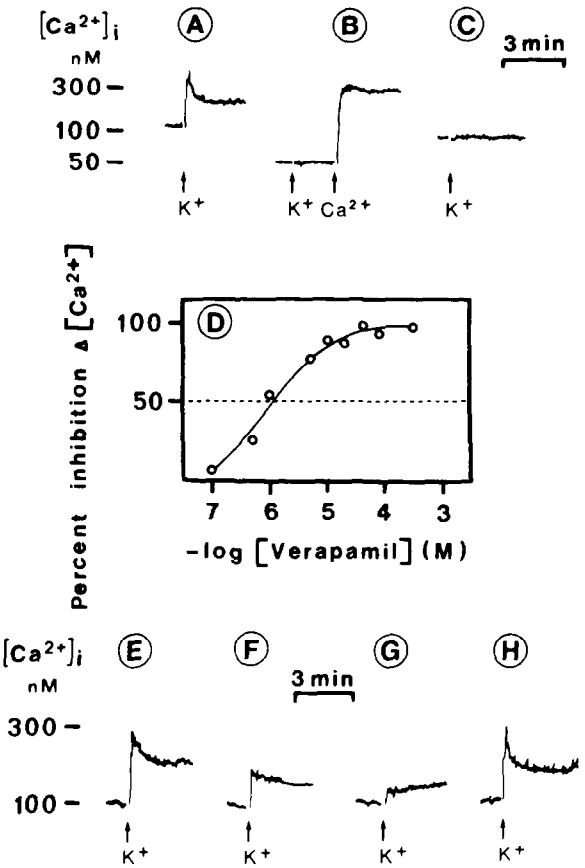

Fig.1. Fura-2 fluorescence traces of the K^+-induced $[Ca^{2+}]_i$ rise in suspended PC 12 cells. For continuous registration the emitted fluorescence signal (500 nm) was recorded only at 340 nm excitation wavelength. Arrows indicate 20 μl additions of K^+ or Ca^{2+} (55 mM and 2 mM final free concentration, respectively). The numbers on the left represent the $[Ca^{2+}]_i$ calibration scale (nM). The bar indicates 3 min. The tracings presented are representative of at least four independent experiments. Panels: A,E, PC 12 cells in complete Ca^{2+} containing incubation medium (controls); B, K^+-depolarization in Ca^{2+} free incubation medium (2 mM EGTA), and after addition of excess Ca^{2+}; C, K^+-depolarization in the presence of 300 μM diltiazem (15 min preincubation) in complete incubation medium; D, dose-response curve for dl-verapamil. Values refer to the height of initial $[Ca^{2+}]_i$ rises above resting levels produced by K^+ at various drug concentrations, and were expressed as percent inhibition as compared with the controls (mean values from three different experiments, SD $<10\%$); F–H, K^+-depolarization in the presence of 2% (F) and 4% (G) halothane, and after washout of 4% halothane by subsequent medium exchange with anesthetic free incubation medium (H).

presence of external Ca^{2+}, since it did not occur in the Ca^{2+} free medium containing 2 mM EGTA. Readdition of Ca^{2+} to the cells challenged with 55 mM K^+ in the absence of external Ca^{2+} resulted in a rapid rise in $[Ca^{2+}]_i$ (fig.1B).

Experiments with prior addition of the Ca^{2+} channel antagonists diltiazem or verapamil (15 min drug exposure, 300 μM each) also indicated that the K^+-evoked $[Ca^{2+}]_i$ rise corresponded to a Ca^{2+} influx through VSCC (fig.1C,D). The concentrations at which verapamil (IC_{50} 1 μM; fig.1D) and diltiazem (IC_{50} 7 μM) reduced the depolarization-induced $[Ca^{2+}]_i$ rise were very similar to those reported previously for $^{45}Ca^{2+}$ uptake through VSCCs into PC 12 cells [13].

All the anesthetics tested in this study inhibited the $[Ca^{2+}]_i$ rise in a dose-dependent fashion (fig.1,

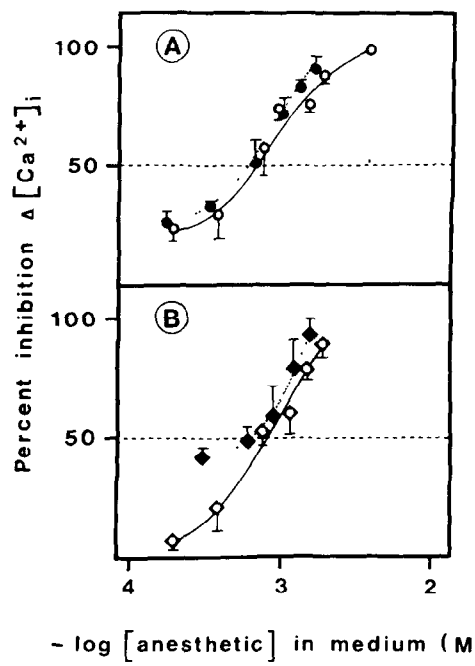

Fig.2. Inhibitory effects of halothane (A, solid circles), methoxyflurane (A, open circles), isoflurane (B, solid diamonds) and enflurane (B, open diamonds) on the $[Ca^{2+}]_i$ rise in PC 12 cells. Values are expressed as percent inhibition compared with controls, and refer to the height of the K^+-induced initial $[Ca^{2+}]_i$ rise above the resting levels. Data represent mean values ± SD of four independent experiments with duplicate determinations. Molar anesthetic concentrations in medium are indicated on the abscissa in a logarithmic scale.

panels E–G; fig.2A,B). As illustrated in fig.1F and G for halothane (2% and 4% in air), the height of the initial $[Ca^{2+}]_i$ peak was especially reduced. Therefore, percent inhibition values plotted on the dose-response curves (fig.2A,B) refer to the height of the K^+-evoked initial $[Ca^{2+}]_i$ rise in the presence of anesthetics. The volatile anesthetics produced half-maximal inhibition at almost identical concentrations in the incubation medium (0.6 mM halothane; 0.58 mM isoflurane; 0.78 mM enflurane; 0.59 mM methoxyflurane). The two curves for halothane and methoxyflurane are nearly superimposable (fig.2A), while the dose-response curve of isoflurane differs at low concentrations from that obtained for its isomer, enflurane, which seems slightly less inhibitory than the other anesthetics (fig.2B). Almost complete depression of the $[Ca^{2+}]_i$ rise was seen beyond 5% vaporized isoflurane, enflurane, halothane and beyond 2% methoxyflurane. Inhibition occurred within 2 min after exposure to the agents and proved to be easily reversible (fig.1H) by medium exchange. External Ca^{2+} was not competitive with the inhibitory effect (not shown), suggesting that volatile anesthetics and classical Ca^{2+} channel antagonists reduce the $[Ca^{2+}]_i$ rise by separate mechanisms [20].

4. DISCUSSION

Changes in $[Ca^{2+}]_i$ can be elicited by mobilizing Ca^{2+} from intracellular stores and/or by increasing the Ca^{2+} permeability of the plasma membrane. In adrenal medulla cells and in PC 12 cells the cholinergic-receptor-mediated Ca^{2+} changes involve both mechanisms in a complex regulatory system that is far from being fully understood [21]. By contrast, in PC 12 the K^+-evoked $[Ca^{2+}]_i$ rise is characterized pharmacologically, biochemically and electrophysiologically as the Ca^{2+} influx through VSCCs [9–13] which are regulated by protein kinase C [19,22,23].

The data reported here document for the first time that the depolarization-evoked $[Ca^{2+}]_i$ rise in a neurosecretory cell line is depressed by volatile anesthetics at clinical concentrations (<1 mM). The depression was a common feature of all the anesthetics tested, and their molar inhibitory potencies were almost identical, suggesting common target sites. That such a uniform inhibition of transmembrane ion transport is not an inevitable characteristic of volatile anesthetics has recently been shown for the Na^+-K^+-Cl^- co-transporter in glial cells, which is competitively inhibited by small molecules like halothane, but not by enflurane, isoflurane or methoxyflurane [24].

The present findings in the PC 12 cell line contrast with recent investigations in cultured bovine adrenal medulla cells that demonstrated an inhibitory effect of halothane on the cholinergic-receptor-mediated, but not on the K^+-induced $^{45}Ca^{2+}$ influx [7]. However, PC 12 cells differ in many aspects from medulla cells, particularly regarding the modulating role of protein kinase C on the VSCC activity. In PC 12 cells the activation of protein kinase C inhibits the $[Ca^{2+}]_i$ rise induced by K^+-depolarization [19,22,23], whereas in rat adrenal medulla cells the depolarization-evoked Ca^{2+} influx is facilitated under similar conditions [25]. Furthermore, even medulla cells differ species-specifically in the relative contributions of muscarinic- or nicotinic-receptor-mediated processes for cholinergic responses [21,26]. Thus, the apparent differences in intracellular Ca^{2+} regulatory mechanisms may account for the discrepancy between our findings and data obtained with bovine medulla cells [7].

In our study the inhibitory effect could not be overcome by increasing external Ca^{2+} concentrations, suggesting that volatile anesthetics may interact with the VSCC in a manner that is different from that of classical Ca^{2+} channel antagonists [20]. An investigation of this phenomenon in a permanent cell line of well defined properties would be of value in further elucidating the interference of volatile anesthetics with the regulation of intracellular free Ca^{2+} levels.

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